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(54) Title: DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATION AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

(57) Abstract

The invention relates to a method for detecting specific target-cells in a simple and time saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. The method can further be used for isolation of the target-cells by magnetic field application and a kit for performing the method according to the invention is described.

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Detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations.

The present invention relates to an immunomagnetic method for detection and positive isolation of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit for performing the method in different cell populations.

In biology, biochemistry and adjacent fields it is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sofisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sofisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen- antibody, enzyme- receptor, ligand- receptor interactions on cells and biotin- avidin binding, of which antigen-antibody binding is most frequently used. A hapten/anti-hapten binding pair method has also recently been suggested (PCT/EP90/01171).

When such methods are used for isolation of specific cells, which are the subject for further various examinations, it is necessary to reverse the linkage without producing destructive effects on the binding partners, which ideally should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for

adequately cleaving antigen/anti-antigen and hapten/anti-hapten linkages (PCT/EP91/00671, PCT/EP90/01171).

Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles or beads, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/-antibody/particle-complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (PCT/EP90/01171) and also a method for isolating haemopoietic progenitor cells from bone marrow (PCT/EP90/02327). The latter is directed to use a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haemalopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles.

PCT/EP90/01171 relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, anti-hapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later

cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

There is a need for a simple linkage to connect a target cell to an insoluble support, which do not involve compounds of a rather unspecified haptene-group, and which is directed to detection of specific target cells and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

Thus the object of the present invention is to detect specific target cells. It represents a sensitive detection method for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and exeedingly simple. Furthermore, the present method can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. A further object of the invention is to provide a kit for performing the method as characterized in the claims.

The intensions of the inventions are obtained by the method and kit characterized in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cells populations is suitable both for detection, and in addition positive isolation of both normal cells and patogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as paramagnetic particles or beads, which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the

membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood.

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cytosentrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and

immunofluorescence. Thus, slides prepared from biopsied tumors or cytosentrifugates have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cellsuspension an use a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flowcytometric examiniations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence (Beiske et al., Am. J. Pathology 141 (3), September 1992). Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a high number of cells can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The monoclonal antibodies used bind specifically to, for example, tumor cells and not to other cells than the target-cells present in the blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC-31), Cluster 2 (MW~40kD) antigen (NrLu10) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insuline receptor, insuline-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells, in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other

disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibody-coated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25°C, preferably at 4°C, under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20°C, preferably 4°C, under gentle rotation. The paramagnetic particles or beads, uncoated or precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25°C, preferably 4°C under gentle agitation. Samples of the cell suspension are then transferred to a cell counting device, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The number of antibody-coated beads added to the cell suspension should be between 2-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells.

For specific purposes, and in the cases where the density of the target-cells is low for example malignant cells, or the target-cells represent a very low fraction of the total number of cells ($\leq$ 1%), the target cells can be positively separated

from non-target cells in a magnetic field. The isolated target cells, can then be enumerated microscopically and the fraction of target cells relative to the total number of cells in the initial cell suspension can be calculated. Moreover, the target-cells may be characterized for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity.

With the existing methods, signals obtained on Southern, Northern and Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerasse chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic

particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc-portion the target-cell associating antibodies, bound to specific anti-target-cell antibodies.

The following examples are given to present a further disclosure of the novel method. These examples shall not be regarded as in any way limiting the invention.

EXAMPLE 1

T47D human breast carcinoma cells were incubated for varying lenghts of time with Hoechst fluoresence dye, and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to 1 x 10^6 bone marrow cells obtained from healthy volunteers. In different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads P450) coated with individual anticarcinoma antibodies (NrLu10, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1-10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2-15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per 10^4 nucleated cells. In control experiments with labeled tumor cells using antibodies known to have some cross-reativity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without

tumor-associated antibody coating, none of the target cells bound any beads.

Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

EXAMPLE 2

Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were sentrifuged, the same coated paramagnetic particles used in Example 1 were added, incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of anibody-coated beads. In some cases, tumor cells were separated in a magnetic field and transferred to tissue culture flasks containing growth medium specially prepared for growing breast cancer cells, in attempts to establish permanent cell lines from these cultures. In parallel, cells from the malignant effusions were cultivated directly without positive selection with magnetic beads. In the latter cases, no cell line could be established, whereas in more than 50 % of the cases where positively selected tumor cells had been used, cell lines were successfylly established.

EXAMPLE 3

In a few cases, bone marrow and peripheral blood obtained from patients with breast cancer were examined with the present procedure by adding antibody-coated paramagnetic beads, incubating for 30 min at 4°C and concentrating in a magnetic field and by examining the suspension under light microscopy. In both cases binding of the paramagnetic beads to 0,1-1 % of the nucleated cells in the bone marrow and blood was detected, cells that could not be identified by any other method.

EXAMPLE 4

Antibodies against certain growth factor receptors or other gene products expressed on the surface of specific cell populations may be used to identify and positively select these cells. Beads coated with anti-transferrin, used in the novel method according to the present invention were shown to represent a rapid, simple and sensitive method for identification of cells expressing the transferrin-receptor.

EXAMPLE 5

For various purposes isolation of specific populations of normal cells is warranted. Endothelial cells lining the capillary or small vessels in normal or tumorous tissue could be positively selected from cell suspensions prepared from the relevant tissues. The procedure involved the use of beads coated with antibody directed against structures expressed on the endothelial cells, but not on the other normal cells in the cell mixture.

EXAMPLE 6

Human cells injected into immunodeficient rodents was shown to be present in cell suspensions prepared from tumor xenografts and from various host organs/tissues by employing magnetic particles coated with an anti-pan human antibody.

CLAIMS

1. Method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, characterized by comprising the following steps:

1-1, coating, by a per ce known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or;
b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and

1-2-1, mixing the target-cell-associating antibodies (murine or human) which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target-associating antibodies, with the cell suspension containing the target-cells, or,

1-2-2, mixing free target-cell-associating antibodies with the cell suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0°C and 20°C, preferably 4°C under gentle rotation, and;

1-3, incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1), or paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2 h, preferably 30

min, at a temperature between 0°C and 25°C, preferably 4°C, under gentle rotation, and;

1-4-1, subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (≤ 1 %) and then examining and counting the target-cells in the cell suspension, using a micropscope and/or a suitable cell/particle counting device, or,

1-4-2, examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3); using a microscope and/or a suitable cell/particle counting device if the ratio of target-cells/total cells in the cell suspension is adequate (> 1 %).

2. Method according to claim 1, characterized by directing the antibody or fragments thereof against the antigens in normal, living cells.

3. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.

4. Method according to one of the preceding claims, characterized by using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insuline receptors, insuline-like receptors transferrin receptor, NGF and FGF receptors.

5. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims, characterized by directing the antibody or fragments thereof against antigen or receptors in cells with abnormal developmental patterns, preferably such as primary and metastatic cancer cells.

7. Method according to one of the preceding claims, characterized by using as the said target-cell associating antibodies, antibodies of the IgG isotype, or $F(ab')_2$ or F(ab) fragments, or IgM, or fragments of IgM.

8. Method according to one of the preceding claims, characterized by preparing the mentioned cell suspension from mixed cell populations comprising mammalian tissues, for examples human bone marrow and peripheral blood, from pleural and peritoneal effusions, other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic gland, skin and mucous membranes.

9. Method according to one of the preceding claims, characterized by that the antibody is directed against groups of antigen determinants, such as CD56/NCAM, for example MOC-1, Cluster 2 epithelicel antigens, for example MOC-31, Cluster 2 (MW~40kD) antigens, for example NrLu10, p97 and HMW-melanoma-associated antigens, for example anti-p97 and 9.2.27, 80kD Sarcoma-associated, for example TP-1 and TP-3, mucin antigens, for example 12H12, and antigen recognized by the Anti-pan-human antibody.

10. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is directed against growth factor receptors and oncogene products expressed on the membrane of malignant cells, for example EGF-receptor, Neu/erb B2/HER2, PDGF (A and B)

receptor, insuline receptors, insuline-like receptors, transferrin receptor, NGF and FGF receptors.

11. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins, other adhesion membrane molecules and MDR proteins in abnormal cells.

12. Method according to one of the preceding claims, characterized by using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.

13. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting magnetically aggregated cells are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level.

14. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established in vitro cell cultures from the separated paramagnetic particletarget-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.

15. Kit for performing the method according to one of the preceding claims, characterized by that it comprises;
1, specific antibody or antibody fragments directed to the antigen receptors on the wanted target-cells, where said

antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or

2, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or

3, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/53, C12N 5/08, C12Q 1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4710472 (SAUR ET AL), 1 December 1987 (01.12.87), examples I and II --	1-15
X	EP, A1, 0395355 (THE JOHNS HOPKINS UNIVERSITY), 31 October 1990 (31.10.90), examples 3-8, 12 and 14	1,2,7,8, 13-15
Y		1-15
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X	PROGRESS IN CLINICAL AND BIOLOGICAL RESEARCH, Volume 333, 1990, C.I. Civin et al, "Positive stem cell selection - basic science", page 387 - page 402, Discussion pages 396-398	1,2,7,8, 13-15
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4920061 (POYNTON ET AL), 24 April 1990 (24.04.90), column 3, line 58 - line 68, example 4	1-3,6-8,12, 13,15
X	WO, A1, 9010692 (UNIVERSITY OF FLORIDA), 20 Sept 1990 (20.09.90), examples 1-3	1,6-8,12,15
X	US, A, 4219411 (YEN ET AL), 26 August 1980 (26.08.80), column 10 - column 15	1,2,7,8,13, 15
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Y	WO, A2, 9007380 (MILTENYI, STEFAN), 12 July 1990 (12.07.90), pages 4,5 and 21-25	1-15
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